Genetic Manipulation of *Borrelia*

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Abstract

Genetic studies in *Borrelia* require special consideration of the highly segmented genome, complex growth requirements and evolutionary distance of spirochetes from other genetically tractable bacteria. Despite these challenges, a robust molecular genetic toolbox has been constructed to investigate the biology and pathogenic potential of these important human pathogens. In this review we summarize the tools and techniques that are currently available for the genetic manipulation of *Borrelia*, including the relapsing fever spirochetes, viewing them in the context of their utility and shortcomings. Our primary objective is to help researchers discern what is feasible and what is not practical when thinking about potential genetic experiments in *Borrelia*. We have summarized published methods and highlighted their critical elements, but we are not providing detailed protocols. Although many advances have been made since *B. burgdorferi* was first transformed over 25 years ago, some standard genetic tools remain elusive for *Borrelia*. We mention these limitations and why they persist, if known. We hope to encourage investigators to explore what might be possible, in addition to optimizing what currently can be achieved, through genetic manipulation of *Borrelia*.

Introduction

Spirochetes in the genus *Borrelia* have been known to cause human disease for well over a century, but in-depth investigations of these pathogenic bacteria awaited many developments, including the advent of molecular genetics. Since transformation of *Borrelia burgdorferi* was first described in 1994 (Samuels et al., 1994b; Samuels, 1995), a molecular genetic approach has been applied with growing success to study the biology and pathogenic potential of these tick-borne spirochetes (Tilly et al., 2000; Cabello et al., 2001; Rosa et al., 2005; Battisti et al., 2008; Fine et al., 2011; Brisson et al., 2012; Raffel et al., 2014; Krishnavajhala et al., 2017; Raffel et al., 2018; Samuels et al., 2018). Both *in vitro* and *in vivo* analyses have thus been conducted to identify and describe the basic functions of many *Borrelia* genes and gene products and their contributions to the relationship of these important pathogens with their tick vectors and mammalian hosts. Direct applications of genetic tools developed for Gram-positive and Gram-negative bacteria have encountered

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limitations, however, due to the genetic distance and distinct codon usage of *Borrelia* relative to these bacteria (Paster et al., 1984; Fraser et al., 1997). Hence there is still a significant need to develop additional and alternative genetic tools for *Borrelia* in particular, and for spirochetes in general.

Over 30 years ago, Dr. Stanley Falkow, the father of the field of microbial pathogenesis, restated Koch's postulates as they could be applied in microbiology using the tools of molecular genetics (Falkow, 1988). In their simplest form, Falkow's molecular postulates entail the ability to create mutations, restore genes and analyze the phenotypes of an otherwise isogenic set of microbes. However, their interpretation and application continue to evolve as new technologies emerge with which to investigate the complex biology of pathogenic microbes (Falkow, 2004; Casadevall, 2017).

Molecular genetic analyses have provided insight into a wide array of regulatory and structural elements of *Borrelia*. However, limitations persist for genetic studies of both the Lyme disease (LD) and relapsing fever (RF) *Borrelia*, including low frequencies and efficiencies of transformation, complex and undefined growth media, and a variable ability to quantify or clone spirochetes as colonies in solid medium. In addition, the generation of isogenic wild-type, mutant and complemented clones present unique challenges in *Borrelia* due to the segmented and unstable nature of their genomes, and poorly defined, multifactorial barriers to the uptake and incorporation of exogenous DNA. Polar effects following insertional inactivation and allelic exchange can arise as a consequence of the compact chromosome of *B. burgdorferi*, which contains many genes with overlapping transcriptional and termination signals (Fraser et al., 1997; Adams et al., 2017b). The presence of paralogous gene families (Casjens et al., 2000) encoding related products with potentially redundant functions in *B. burgdorferi* can also complicate the interpretation of mutant phenotypes. Establishing the connection between a mutation and a phenotype through complementation is essential, but it can be particularly challenging. Isolation of nutritional mutants in *Borrelia* is limited by the complex and undefined nature of the growth media and a relatively long doubling time (>5 hours under optimal conditions) (Barbour, 1984). Despite these potential and real challenges, substantial progress has been made in utilizing genetic approaches to study factors involved in the biology and physiology of *Borrelia* and pathogenesis of the diseases they cause, as illustrated elsewhere (Radolf and Samuels, 2021).

Clone and strain considerations

Lyme disease spirochetes

Most genetic studies to date have been conducted with *B. burgdorferi* sensu stricto (ss) strains, with only a few studies utilizing *B. afzelii* or *B. garinii* strains (Fingerle et al., 2007; Bontemps-Gallo et al., 2018; Cuellar et al., 2019; Strnad and Rego, 2020). Initially, the complete and annotated genomic sequence, including all plasmids, was only available for *B. burgdorferi* ss type strain B31 (Fraser et al., 1997; Casjens et al., 2000), which made it the obvious choice for genetic studies. However, this limitation no longer exists, and the sequences for the complete genomes, including plasmids, are either available or readily determined for virtually any *Borrelia* isolate (Casjens et al., 2017; Casjens et al., 2018).

Sequencing advances that yield extremely long and accurate reads from single DNA molecules have been key to this development (Margos et al., 2017). The linear chromosome is highly conserved other than a short region at one telomere, whereas the plasmid component of the genome has undergone substantial rearrangement among strains. However, the total gene content of *B. burgdorferi* ss is relatively constant, with few absolute differences among the large number of isolates analyzed to date, despite widespread plasmid reorganization (Casjens et al., 2017). The variability in plasmid structure among strains suggests that the phenotype of a particular mutant (role/contribution of a particular gene) could vary with the context (strain background) in which it is analyzed. Ideally, mutations would be introduced and analyzed in more than a single clone or strain (Gilbert et al., 2007; Lybecker and Samuels, 2007). However, recognizing that experimental outcome may differ between strains and interpreting results accordingly is currently acceptable, given the limitations and challenges presented by genetic studies in *B. burgdorferi*.

Strain choice by individual labs generally reflects what has been used in previous experiments to facilitate comparison with existing data. There are no data indicating that a particular LD spirochete strain is inherently more suitable than another. However, the ease with which spirochetes can be grown and plated, the efficiency at which they can be transformed, and their phenotype in the mouse/tick infectious cycle are relevant traits that vary substantially among *B. burgdorferi* strains and between clones derived from a single strain (Elias et al., 2002). Hence a key consideration at the outset of any experiment is whether characteristics of the clone/strain/genospecies being used, such as those described above, are appropriate for the question(s) being asked.

Although differences in plasmid content (and corresponding differences in plasmid-borne restriction/modification systems) clearly contribute to the efficiency with which different LD strains and clonal derivatives can be transformed (Lawrenz et al., 2002; Kawabata et al., 2004; Rego et al., 2011), additional traits also affect transformability. These include surface properties that permit or restrict resuspension in electroporation solution (Tilly et al., 2000; Tilly et al., 2016). The highly segmented and potentially unstable structure of the *B. burgdorferi* genome mandates that plasmid content be continuously monitored to assure that mutant and wild-type clones remain isogenic except for directed genetic changes (Elias et al., 2002). Determining the plasmid content of strain B31 derivatives has been greatly facilitated by development of multiplex PCR methods (Bunikis et al., 2011; Norris et al., 2011). Complementation also represents a critical component of genetic experiments in *Borrelia*. Finally, it is important to remember that a wild-type *Borrelia* "clone" is neither clonal nor isogenic after passage through a mouse, due to rampant recombination at the *vlsE* and *vmp* expression loci of LD and RF spirochetes, respectively (Stoenner et al., 1982; Barbour, 1990; Restrepo and Barbour, 1994; Zhang et al., 1997; Zhang and Norris, 1998; Coutte et al., 2009; Verhey et al., 2019). Spirochetes isolated from a mouse comprise a complex population of genetically and phenotypically distinct bacteria expressing different alleles of these abundant and immunodominant surface proteins. This heterogenous population of spirochetes recovered from a mouse can serve as the source from which to derive a new infectious clone by plating or limiting dilution, but it should not be used directly as the starting material for genetic manipulation.

Transformation protocol

B. burgdorferi was first genetically transformed in 1994 by electroporation, following a previous study in which the effect of buffers and electroporation parameters on *B. burgdorferi* had been tested (Sambri and Lovett, 1990; Samuels et al., 1994a). A detailed protocol for electro-transformation of *B. burgdorferi* was published by Samuels et al. in 1995 (Samuels, 1995) and recently updated (Samuels et al., 2018). Although increasingly sophisticated genetic tools and techniques have become available, the only significant improvement to the original protocol is the addition of large amounts of DNA (10–50 μg) as electroporation substrate (Tilly et al., 2000). More recently, recognizing and avoiding barriers presented by plasmid-borne restriction/modification (R/M) genes (Lawrenz et al., 2002; Kawabata et al., 2004; Jewett et al., 2007a; Chen et al., 2008; Rego et al., 2011; Kasumba et al., 2015), and routinely monitoring total plasmid content of transformants by multiplex PCR (Bunikis et al., 2011; Norris et al., 2011) have permitted genetic manipulation of fully infectious *B. burgdorferi* clones, which is needed for *in vivo* studies.

Transformation frequency and efficiency

Two calculations are typically used to monitor how well a transformation has worked. Each provides important information for developing, optimizing or applying genetic tools to *Borrelia* and should be used routinely. The first, termed transformation frequency, describes what fraction of the population that survives electroporation (or other transformation method) has stably acquired the introduced DNA. This is determined by quantifying viable bacteria in comparable aliquots of the post-electroporation culture, with and without selection for the transforming DNA. The other standard calculation is transformation efficiency, which is determined by calculating the number of transformants obtained per microgram of DNA used in the electroporation. Transformation frequency and efficiency can be determined only if selection is imposed and viable bacteria are quantified shortly after recovery from electroporation, before the surviving bacteria have undergone substantial cell division. Accurate determinations of transformation frequency and efficiency also require verification that bacteria growing under selection are transformants and not spontaneous resistance mutants. The frequency and efficiency of transformation in *Borrelia* are notoriously poor, which means that a large number of spirochetes and many micrograms of DNA are needed per electroporation in order to recover a transformant. In general, high passage, non-infectious *B. burgdorferi* spirochetes are more readily transformed (higher frequency and efficiency) than low-passage infectious clones; this is due primarily to the loss of plasmids carrying R/M genes, but other poorly understood factors also contribute.

Preparation of competent cells

The preparation of electrocompetent *Borrelia* involves growing spirochetes to a suitable density, followed by a series of washes to concentrate cells and remove culture medium, especially salts, which cause arcing, cell death, and, of course, decreased transformation efficiency (Samuels et al., 1994a; Samuels, 1995; Samuels et al., 2018). Cell density and growth phase are important criteria to consider for successful electrotransformation of *Borrelia* (Samuels, 1995; Tilly et al., 2000; Hübner et al., 2001). Spirochetes in mid-log phase have the highest transformation frequency, but competent cell preparations from late-

log phase cultures, which have a greater number of cells, yield the most transformants (Samuels, 1995; Tilly et al., 2000). Briefly, cells are harvested sterilely by centrifugation, washed several times, and finally resuspended in cold electroporation solution as an approximately 100-fold concentrated cell slurry. Some *B. burgdorferi* strains aggregate during the washes and cannot be resuspended in electroporation solution, which prevents or severely limits transformation (Tilly et al., 2000); this undesirable trait is intensified when bacteria are grown to higher cell density. The presence of outer surface protein B (OspB) contributes to this clumping phenotype (Tilly et al., 2016); hence most strains selected for use in genetic studies are spontaneous mutants that lack OspB (Elias et al., 2002). Aliquots of competent cells can be transformed by electroporation immediately or they can be stored at −80°C, which decreases transformation efficiency by about 50% (Samuels, 1995; Samuels and Garon, 1997; Samuels et al., 2018).

Electrotransformation

Electroporation conditions for transformation of *B. burgdorferi* are similar to those used for other bacteria (Samuels et al., 1994a; Samuels, 1995; Samuels et al., 2018). A high-voltage electric pulse generates pores in the cellular membranes through which DNA can enter the cytoplasm (Shigekawa and Dower, 1988; Trevors et al., 1992; Nickoloff, 1995). Transmission electron microscopy of electroporated *B. burgdorferi* reveals darkly stained regions on the membrane along the length of the cell that are thought to be disruptions in the surface (Samuels and Garon, 1997). These putative pores are no longer visible after a 30 minute incubation in culture medium.

As mentioned above, a significant improvement to the original protocol was increasing by approximately 1000-fold the amount of DNA used per electroporation, from the nanogram levels typically used with other bacteria, to the microgram quantities needed to routinely transform *B. burgdorferi* (Bono et al., 2000; Tilly et al., 2000; Hübner et al., 2001; Stewart et al., 2001; Eggers et al., 2002; Elias et al., 2002). However, even when successful, relatively few *B. burgdorferi* transformants are typically recovered per electroporation, particularly when working with infectious clones. Aside from R/M systems, which can be eliminated, the basis for the extremely low transformation frequency and efficiency of *B. burgdorferi* remains a mystery. Perhaps very little of the transforming DNA actually gets into the cell, the DNA is rapidly targeted for degradation once it enters, or some other feature of the spirochete limits stable retention of transforming DNA. Whatever the basis, this unfortunate characteristic limits the routine construction of saturated libraries that are needed to apply powerful forward genetic screens in *Borrelia* (Lin et al., 2012; Ellis et al., 2013).

Transformation substrate

In addition to the amount of DNA, its source and form have an effect on transformation efficiency. Allelic exchange transformation was first demonstrated by introducing site-directed mutations that conferred antibiotic resistance, using PCR-generated linear DNA substrates (Samuels et al., 1994a). Short oligonucleotides also can be used as transformation substrates (Samuels and Garon, 1997), although this has limited utility because the site-directed mutation encoded by the oligonucleotide must confer a selectable advantage (currently limited to antibiotic resistance). A circular suicide plasmid was previously shown

to recombine into the genome at the same frequency as linear DNA (Stevenson et al., 1998); hence linearized plasmid DNA or PCR-amplified DNA fragments are typically used when allelic exchange is the desired outcome. This prevents integration of suicide plasmids and generation of merodiploids, which are recovered more frequently when working with *Borrelia* clones lacking loci encoding restriction-modification (R/M) systems. As described in a later section, circular shuttle vectors have been constructed that replicate autonomously in *B. burgdorferi* and *E. coli* (Stewart et al., 2001; Eggers et al., 2002; Stewart et al., 2003; Byram et al., 2004). In addition, linear shuttle vectors have been constructed using replication functions from both native circular and linear plasmids (Chaconas et al., 2001; Beaurepaire and Chaconas, 2007). Transformation of autonomously replicating shuttle vectors into strains lacking R/M systems (see below) is generally more efficient than that of allelic exchange constructs (Elias et al., 2003), presumably because the latter require the additional step of recombination. Remarkably, intact native plasmids up to 36 kbp in length have been successfully transformed into *B. burgdorferi*. (Grimm et al., 2004; Jewett et al., 2007b).

The requirement for microgram quantities of substrate DNA per *Borrelia* transformation can present a problem in itself. Plasmids containing *Borrelia* sequences or *Borrelia* codon-optimized genes can limit *E. coli* growth, and rapid propagation of *E. coli* to high cell densities (as typically done for large scale plasmid preparations) can select for mutations in sequences conferring a growth phenotype. Propagating *E. coli* carrying recombinant plasmids at a lower temperature, such as 30°C, (Takacs et al., 2018), lessens the selection for mutations that confer a growth advantage, and plasmid DNA preparations should be sequenced to confirm the integrity of the insert.

DNA modification

One of the identified barriers to efficient transformation of *B. burgdorferi* is the presence of plasmid-encoded restriction-modification (R/M) systems (Lawrenz et al., 2002; Kawabata et al., 2004). Transformation of a set of closely related *B. burgdorferi* strain B31 clones with distinct plasmid complements demonstrated that loss of two linear plasmids, lp25 and lp56, correlated with higher transformation efficiency of a shuttle vector (Lawrenz et al., 2002). These two plasmids carry genes *(bbe02* and *bbq67)* that are predicted to encode type IV R/M enzymes. Disruption of *bbe02* increased shuttle vector transformation efficiency approximately 40-fold (Kawabata et al., 2004). Subsequent studies confirmed these initial observations and demonstrated that *bbe02* and *bbq67* of strain B31 encode distinct R/M enzymes that methylate endogenous DNA and restrict foreign DNA lacking sequence-specific modifications (Chen et al., 2008; Rego et al., 2011). The motifs modified by *bbe02* and *bbq67* were recently inferred by SMRT sequence analysis of genomic DNA from B31 clones that differed in R/M gene content (Casselli et al., 2018). This information should facilitate the design of optimized constructs for more efficient transformation of B31 clones containing intact *bbe02* and *bbq67* loci. Analysis of genomic sequences in the database indicates that some *B. burgdorferi* strains contain additional R/M loci that likely recognize and modify novel sequence motifs (Rego et al., 2011). However, a third plasmid-borne R/M locus in strain B31, *bbh09* on lp28–3, does not negatively impact shuttle vector

transformation (Lawrenz et al., 2002; Kawabata et al., 2004), suggesting that the presence of R/M loci does not necessarily indicate a barrier to transformation in these strains.

In addition to the *bbe02* R/M locus, lp25 also carries genes required for mouse and tick infectivity (Purser and Norris, 2000; Labandeira-Rey and Skare, 2001; Purser et al., 2003; Grimm et al., 2005; Revel et al., 2005; Strother et al., 2005; Gilmore et al., 2014). The linkage of these disparate functions on lp25 is the primary reason that infectious B31 derivatives (containing lp25) are difficult to genetically transform. Transformation efficiency into low-passage infectious strains can be increased by first transforming the DNA into a strain carrying lp56 but lacking lp25 (Jewett et al., 2009). The resulting genomic DNA purified from *Borrelia* is expected to be partially modified and thus presents less of an obstacle when introduced in subsequent transformations into strains containing lp25. The transformation barrier imposed by putative restriction enzymes on lp56 and lp25 applies primarily to shuttle vector transformations rather than gene inactivation by allelic exchange (Elias et al., 2002; Lawrenz et al., 2002; Hübner et al., 2003; Kawabata et al., 2004; Tilly et al., 2004). This likely reflects the requirement for intact circular DNA for autonomous plasmid replication, whereas linearized DNA remains an effective template for allelic exchange. Consequently, *trans*-complementation on a shuttle vector presents a greater challenge than gene inactivation in infectious clones retaining lp25.

Fortunately, spirochetes do not require *bbe02* or *bbq67* for growth *in vitro* or at any stage of the experimental mouse/tick infectious cycle. Therefore, fully infectious B31 variants have been constructed in which the *bbe02* locus on lp25 has been deleted (Lawrenz et al., 2002; Kawabata et al., 2004; Rego et al., 2011). These modified strains typically lack the (non-essential) lp56 plasmid that carries *bbq67*, thereby further enhancing their suitability for genetic studies. As an added bonus, the presence of an antibiotic resistance cassette in *bbe02* provides selection for lp25 during *in vitro* growth and genetic manipulation. The *bbe02* locus on lp25 has been chosen as an optimal site for insertion of reporter genes, components of inducible systems, and *trans*-complementation (Gilbert et al., 2007; Chan et al., 2015; Kasumba et al., 2015), as targeted insertion at this site guarantees stable retention of introduced DNA at normal copy number throughout the mouse/tick infectious cycle. This is discussed more fully in subsequent Complementation and Reporter gene sections.

Alternative methods for transformation

In addition to electroporation, *B. burgdorferi* has been successfully transformed using a simple protocol that entails resuspension of bacteria and DNA together in a solution of PEG and DMSO (Chung et al., 1989; Elias et al., 2002). However, this method has not been widely used or optimized, and its general utility remains unexplored. Likewise, Eggers and colleagues have demonstrated that the endogenous prophage of *Borrelia* can mediate relatively efficient horizontal gene transfer between co-cultured spirochetes (Eggers et al., 2016), but phage transduction has not been developed as a standard genetic tool for *B. burgdorferi*. A substantial advantage of both protocols over electroporation is that they require fewer bacteria at the outset of an experiment because neither technique results in significant cell death, thereby greatly reducing (~100-fold) the initial culture volume needed per transformation.

Selectable markers

Antibiotic resistance markers are one of the most effective selective tools used to single out rare bacterial variants containing new genetic properties, as well as to select for and screen mutants deficient in the expression of specific genes (Pich et al., 2006). *B. burgdorferi* is exquisitely susceptible to many antibiotics (Hunfeld et al., 2002; Ruzic-Sabljic et al., 2005; Hunfeld and Brade, 2006), but the choice of antibiotic resistance genes as genetic markers is somewhat limited by biosafety considerations: markers encoding resistance to ampicillin and tetracycline are prohibited for use in *B. burgdorferi* because these antibiotics are clinically useful in the treatment of Lyme disease (Steere, 2001; Wormser, 2006). The selectable markers currently available for genetic studies in *B. burgdorferi* and their relevant features are listed in Table 1.

Endogenous genes

To date, only antibiotic resistance has been used to select *Borrelia* transformants (Samuels et al., 1994a; Bono et al., 2000; Sartakova et al., 2000; Elias et al., 2003; Frank et al., 2003). An allele of the endogenous *B. burgdorferi gyrB* gene (Samuels et al., 1994b), which encodes the B subunit of DNA gyrase, was the first selectable marker used in *Borrelia* (Samuels, 2006). However, the *gyrB301* marker, which confers resistance to the antibiotic coumermycin A_1, has limited utility for several reasons, including pleiotropic effects due to relaxed DNA supercoiling (Alverson and Samuels, 2002; Alverson et al., 2003). Nonetheless, the use of *gyrB301* was seminal in the development of transformation and genetic manipulation of *B. burgdorferi* (Samuels et al., 1994a; Rosa et al., 1996).

Hybrid genes

A major breakthrough in genetic analysis of *B. burgdorferi* was the construction of hybrid genes using native *B. burgdorferi* promoters fused to foreign open reading frames. This was first used to assay transcription by fusing various borrelial promoters to *cat*, encoding chloramphenicol acetyl transferase (Sohaskey et al., 1997). Bono et al. (Bono et al., 2000) constructed the first efficient selectable marker for *Borrelia* by fusing the strong and constitutive *flgB* and *flaB* promoters of *B. burgdorferi* to the *aphI* gene from Tn*903*, which confers resistance to the aminoglycoside kanamycin; the resulting cassette confers kanamycin resistance in both *E. coli* and *Borrelia*. Several other chimeric genes were constructed in a similar fashion, notably selectable markers conferring resistance to gentamicin with the *aacC1* gene from Tn*1696* (Elias et al., 2003) and resistance to spectinomycin and streptomycin with the *aadA* gene from *Shigella flexneri* plasmid R100 (Frank et al., 2003). Most recently, this approach was applied to the *hph* gene of *E. coli* and the *bsd* gene of *Aspergillis terreus*, yielding codon-optimized selectable markers fused to the *flgB* promoter that confer resistance to hygromycin and blasticidin, respectively (Takacs et al., 2018). Although seemingly straightforward, not all antibiotic resistance cassettes constructed in this fashion function as selectable markers in *B. burgdorferi*. The *cat* and *pac* genes, fused to the *flgB* promoter, do not confer resistance to chloramphenicol and puromycin, respectively (Elias et al., 2003). Hybrid cassettes conferring resistance to various antibiotics and used as selectable markers in *B. burgdorferi* are summarized in Table 1.

Selection parameters

Transformants were originally isolated (Samuels et al., 1994a) as sub-surface colonies in semi-solid medium using a modification (Rosa and Hogan, 1992) of the plating protocol described by Kurtti *et al.* (Kurtti et al., 1987). Following electroporation, cells are allowed to recover in liquid culture for approximately 18 hours in the absence of selection before plating in semi-solid medium containing the selective agent and incubating in a humidified microaerobic atmosphere, typically a CO_2 incubator. Stably transformed bacteria form colonies in the semi-solid medium in five days to two weeks and can be isolated by aspirating a plug of agarose containing the colony to liquid medium. A PCR-screen for the presence of the selectable marker can be used to distinguish transformants from spontaneous antibiotic resistant mutants.

A method of selecting for clonal transformants by limiting dilution was developed by Yang *et al.* (Yang et al., 2004), who were unable to isolate *ospA* mutants in solid medium. Recovered transformants are diluted into 100 ml of liquid medium with selection and distributed among several 96-well plates. Again, the plates are incubated in a humidified atmosphere containing CO_2 and positive wells are detected by the change in color of the medium from red to yellow, which is due to the production of lactic acid by the bacteria during growth, as detected by phenol red in the medium. Putative transformants can be expanded in a larger volume of medium containing antibiotic for confirmation and subsequent analysis. Regardless of the method used to isolate transformants, there is the possibility that two spirochetes are intertwined and thus a colony in semi-solid medium or a positive well at the endpoint of limiting dilution may not be derived from a single cell; therefore, extensive mixing, vortexing, and/or pipetting is required to separate cells before plating.

Not currently available

Effective counter-selectable markers have not been widely applied to *B. burgdorferi*. The use of the *rpsL* gene for counter-selection in *Borrelia* has been described but not utilized in any genetic studies to date (Drecktrah et al., 2010). The lack of defined culture medium, coupled with an inherently limited metabolic capability, has impaired the development of auxotrophic selection as an *in vitro* genetic tool for *Borrelia*. However, Richards and colleagues recently demonstrated that *B. burgdorferi* mutants lacking genes encoding the initial enzymes in acetate utilization are non-infectious in mice and have an absolute requirement for the addition of mevalonate to culture medium, suggesting a means of auxotrophic selection *in vitro* and potential utility as selectable markers *in vivo* (Richards et al., 2015).

Gene inactivation

A number of reviews have outlined various strategies and provided detailed protocols for gene inactivation in *Borrelia* (Hyde et al., 2011b; Drecktrah and Samuels, 2018; Latham and Blevins, 2018; Radolf and Samuels, 2021; Samuels et al., 2018), and this information will not be reiterated in detail in this review. As described above, targeted gene inactivation by homologous recombination is straightforward but inefficient in *B. burgdorferi*, requiring

microgram quantities of DNA and ranging in frequency from 10^{-5} to 10^{-8}, depending upon the recipient organism (Samuels et al., 1994a; Tilly et al., 2000; Elias et al., 2002). Fortunately, illegitimate recombination (insertion of transforming DNA at a non-homologous site) does not commonly occur in *B. burgdorferi* and thus does not impede the recovery of intended mutants. Simple gene disruption can be accomplished by integration (through a single crossover event) of a circular suicide vector carrying an appropriate selectable marker and a fragment of homologous DNA. Alternatively, if homologous sequences flank the selectable marker in the inactivation construct, allelic exchange can occur with the targeted gene through a double crossover event. Depending upon the design of the inactivation construct, allelic exchange can result in simple disruption or deletion of the targeted gene. A somewhat more elegant form of allelic exchange entails modifying the coding sequence of the targeted gene rather than deleting it, to assess the resulting mutant phenotype. This approach has been used for site-directed mutagenesis of a number of *B. burgdorferi* genes (Knight et al., 2000; Yang et al., 2003; Earnhart et al., 2010; Earnhart et al., 2011; Drecktrah et al., 2013).

As mentioned previously, a single crossover event can occur with allelic exchange constructs if they are introduced as circular DNA. This can result in the unintentional introduction into *B. burgdorferi* of other antibiotic resistance markers present on the cloning vector. As stated above, some selectable markers commonly used in *E. coli*, such as those conferring resistance to ampicillin and tetracycline, are not permitted for genetic studies in *B. burgdorferi* due to the clinical utility of these antibiotics in treating Lyme disease. At the minimum, cloning vectors carrying these markers should be linearized prior to transformation into *B. burgdorferi*. Preferably, allelic exchange constructs should be cloned into vectors that do not contain such restricted markers or the genes encoding them should be inactivated prior to transformation of *B. burgdorferi*.

Several approaches for engineering large deletions in *Borrelia* have been described. Chaconas and colleagues co-opted an endogenous cellular activity involved in the formation of telomeres in *Borrelia* to engineer staggered deletions from either end of a linear plasmid to targeted internal sites (Beaurepaire and Chaconas, 2005; Bankhead and Chaconas, 2007). Bestor et al. have adapted the Cre-*lox* site-specific recombination system to *Borrelia* and used it to generate large internal deletions on a linear plasmid (Bestor et al., 2010). Finally, a basic understanding of the genes/sequences required for autonomous replication and plasmid incompatibility has been applied to displace entire plasmids in *Borrelia* (Stewart et al., 2001; Eggers et al., 2002; Stewart et al., 2003; Byram et al., 2004; Stewart et al., 2005; Jewett et al., 2007a; Jewett et al., 2007b; Dulebohn et al., 2011; Dulebohn et al., 2013).

Conditional mutants, as described elsewhere in this review, can now be generated with any of several inducible systems available for *Borrelia*, (Blevins et al., 2007; Gilbert et al., 2007; Whetstine et al., 2009; Latham and Blevins, 2018) Conditional mutants provide an important means to assess the contributions of genes that encode essential functions or for which unmodulated expression is lethal. Finally, all forms of gene inactivation or modification in *Borrelia* entail insertion of a selectable marker at a targeted site, with potential polar effects on adjacent sequences. Interrupted or enhanced transcription of upstream or downstream sequences following insertion of a constitutively expressed antibiotic resistance cassette is

not uncommon and should be considered when designing gene inactivation constructs and interpreting experimental outcome. Promoter-less resistance cassettes have been used successfully within constitutive operons of *B. burgdorferi* to target individual genes without impacting downstream genes (Motaleb et al., 2011; Showman et al., 2016).

Complementation

Complementation plays a crucial role in genetic studies of *B. burgdorferi* due to the extremely low frequency with which targeted gene inactivation occurs and the inherent instability of the segmented genome, as described above. Although measures can be taken to ensure that wild-type and derivative clones contain the same plasmids (Purser and Norris, 2000; Labandeira-Rey and Skare, 2001; Eggers et al., 2002; Elias et al., 2002; Parveen et al., 2006; Bunikis et al., 2011; Norris et al., 2011), screening for more subtle genomic alterations elsewhere in the genome is not routinely feasible. To control for unforeseen or unidentified second site mutations, or polar effects on flanking sequences, the goal of complementation is to leave the original mutation unaltered, but to re-introduce a wild-type copy of the targeted gene in *trans* on an autonomously replicating shuttle vector or elsewhere in the genome, or in *cis* by allelic replacement. As described below, various technical considerations, such as copy number, DNA supercoiling, genomic organization and placement of the antibiotic cassette, should be considered when selecting which complementation approach to use.

Endogenous plasmid shuttle vectors

A number of shuttle vectors have been derived from the endogenous plasmids of *B. burgdorferi*. As reviewed in Radolf and Samuels (2021) the complete genome sequence of *B. burgdorferi* identified a set of paralogous gene families (PGFs) present on all plasmids, suggested to be involved in plasmid replication and maintenance, although lacking strict sequence homology to plasmid genes with comparable functions in other bacteria (Zückert and Meyer, 1996; Casjens et al., 2000). An early shuttle vector derived from a small, non-essential endogenous circular plasmid (cp9) of *B. burgdorferi* demonstrated that the region encompassing these PGFs did not support replication in *E. coli*, but was sufficient for autonomous replication in *B. burgdorferi* (Stewart et al., 2001). This shuttle vector, termed pBSV2, and its derivatives have become the workhorse for plasmid-based *trans*-complementation and other applications in *B. burgdorferi*. In the absence of strong counterselection (Tilly et al., 2006), pBSV2 is relatively stable both *in vitro* and *in vivo* (Stewart et al., 2001), and present at approximately 5–10 copies per cell (Tilly et al., 2006). However, sequences inserted on a shuttle vector (such as for complementation or fluorescent protein expression) can destabilize the shuttle vector, leading to its loss if antibiotic selection is not maintained. Depending on the mutation, the increased copy number of a wild-type gene on the shuttle vector has the potential for ineffective complementation due to deleterious effects of overproduction of the target protein (Sartakova et al., 2001; Eggers et al., 2002; Tilly et al., 2006; Ristow et al., 2012; Kasumba et al., 2015). Additional shuttle vectors have been derived from other endogenous plasmids (lp25, lp28–1, cp26, and a cp32) by cloning their PGF counterparts together with a functional selectable marker (Eggers et al., 2002; Stewart et al., 2003; Byram et al., 2004; Beaurepaire and Chaconas, 2005). Along

with autonomous replication, the PGF regions also mediate incompatibility, resulting in displacement of the endogenous *B. burgdorferi* plasmid from which they were derived (Stewart et al., 2001; Eggers et al., 2002; Stewart et al., 2003; Beaurepaire and Chaconas, 2005), unless selection exists for the coexistence of both replicons (Byram et al., 2004). Displacement with incompatible shuttle vectors thus has been used to experimentally manipulate plasmid content in *B. burgdorferi* (Stewart et al., 2003; Byram et al., 2004; Grimm et al., 2004; Jewett et al., 2007a; Jewett et al., 2007b; Dulebohn et al., 2011; Dulebohn et al., 2013).

Genetic reconstitution at the original locus

An alternative complementation strategy is genetic reconstitution, in which the mutated copy of the gene is replaced with a wild-type copy and another closely linked marker (Hübner et al., 2001; Tilly et al., 2001; Li et al., 2007b), similar to the strategy used to introduce point mutations (Knight et al., 2000; Yang et al., 2003). This approach, however, does not leave the original mutation unaltered. A potential advantage to complementation by genetic reconstitution, at the site of the mutated locus or elsewhere on the same replicon, is that the reintroduced wild-type gene copy is at the same copy number and similar supercoiling state as the original gene. Some attempts to complement mutations in *trans* on a shuttle vector have only partially or unsuccessfully restored the wild-type phenotype (Sartakova et al., 2001; Ristow et al., 2012), raising questions about whether the context of the complementing gene has significantly altered its expression. Alternatively, such results could indicate the presence of an unrecognized second site mutation, which is responsible for the defective phenotype of the complemented mutant. Distinguishing among the possible causes of unsuccessful complementation is addressed below.

Trans-complementation in the genome

An improvement over *trans*-complementation on a shuttle vector is to insert the complementing gene elsewhere in the genome, such as on an essential plasmid (cp26, lp25, lp28–1 or lp36) (Byram et al., 2004; Kawabata et al., 2004; Grimm et al., 2005; Blevins et al., 2007; Gilbert et al., 2007; Jewett et al., 2007b; Caimano et al., 2019). This approach leaves the original mutation undisturbed and maintains *in vivo* selection for the reintroduced gene by virtue of insertion on an essential plasmid. A logical target for this approach is the *bbe02* locus on lp25 as the insertion site. Currently, many research labs employ *B. burgdorferi* clones in which *bbe02* has been inactivated as a means to increase transformation efficiency, yet maintain infectivity (Kawabata et al., 2004; Jacobs et al., 2006; Rego et al., 2011). Further, because lp25 is required for *B. burgdorferi* survival throughout the enzotic cycle, this provides biological selection for *in vivo* maintenance of the complementing gene. The allelic exchange suicide vectors pKBE-Strep and pKBE-Kan have been engineered with a multiple cloning site for insertion of the complementing gene upstream of a linked antibiotic cassette, and flanking DNA homologous to sequences upstream and downstream of the *bbe02* R/M locus (Kasumba et al., 2015). Validation of this approach via complementation of an *ospC* mutant demonstrated copy number on lp25 consistent with the copy number of the native gene on cp26, as well as functional and stable maintenance of the *ospC* gene in *trans* on lp25 (Kasumba et al., 2015). Similarly, the intergenic region between oppositely transcribed *bb0445* and *bb0446* genes on the

chromosome has been used successfully for *trans*-complementation (Li et al., 2007a; Zhang et al., 2009). Innocuous sites on lp25, lp28–1 and lp36 have been useful for insertion of an antibiotic resistance cassette to provide antibiotic selection to ensure *in vitro* maintenance (Blevins et al., 2007) or to restore to *B. burgdorferi* clones lacking each of the critical virulence plasmids (Grimm et al., 2004; Jewett et al., 2007b). Given that this approach effectively preserved or reestablished the wild-type infection phenotype, these sites like *bbe02* on lp25, provide preferable alternatives for stable *trans*-complementation for *in vivo* studies.

Limitations, failures and alternatives

Complementation, while critical to demonstrating the link between a mutant phenotype and gene, presents a substantial challenge when working with infectious *B. burgdorferi* clones retaining the full set of endogenous plasmids. As mentioned above, this is primarily due to the activities of restriction enzymes encoded by genes *bbe02* and *bbq67* on lp25 and lp56, respectively (Lawrenz et al., 2002; Kawabata et al., 2004). The DNA of *B. burgdorferi* contains N^6-methylated adenine only when *bbe02* and/or *bbq67* are present, indicating that both genes encode adenine methyltransferase activity (Rego et al., 2011), and recently the sequence motifs recognized by these restriction enzymes were identified (Casselli et al., 2018). Two genetic approaches to address the barrier that these R/M loci present for transformation of infectious *B. burgdorferi* are (i) to use a genetic background that lacks lp56 and gene *bbe02* (Kawabata et al., 2004; Jacobs et al., 2006; Rego et al., 2011; Jain et al., 2012; Showman et al., 2016; Aranjuez et al., 2019), or (ii) to perform genetic manipulations in a non-infectious, yet transformable, *B. burgdorferi* genetic background lacking lp25 and subsequently restore the *in vivo*-essential lp25-encoded *pncA* (*bbe22*) gene prior to mouse infection studies (Labandeira-Rey and Skare, 2001; Labandeira-Rey et al., 2003; Purser et al., 2003; Seshu et al., 2006; Weening et al., 2008). However, this second approach does not allow tick studies due to the requirement for lp25-encoded genes in addition to *pncA* for tick colonization and persistence (Grimm et al., 2005; Revel et al., 2005; Strother et al., 2005; Gilmore et al., 2014).

When repeated attempts to obtain an isogenic complemented mutant retaining essential plasmids are unsuccessful, an argument can be made linking a mutant phenotype and targeted gene if several independently derived mutants, obtained in separate transformation experiments, are compared with an isogenic wild-type clone. This argument is based on the low probability that second site mutations resulting in a similar mutant phenotype would have arisen independently in separate transformations. Occasionally, a quandary arises when a wild-type copy of the gene has been reintroduced successfully into the mutant strain, yet the defective phenotype persists, leading to the conclusion that complementation has failed. As discussed above, one explanation is that the complementing gene may not be appropriately expressed in *trans* due to changes in supercoiling, copy number or absence of key regulatory sequences. This issue may be addressed by employing one of the alternative complementation strategies. If persistence of the mutant phenotype in the complemented strain cannot be explained by dysregulated gene expression, the next possibility to investigate is polar effects of the original mutation on the transcription of adjacent sequences. If expression of adjacent transcripts appears to be altered by the mutation, then a

different gene inactivation strategy should be employed that minimizes potential polar effects. Finally, if the complementing gene is intact and appropriately expressed, and there is no evidence that the mutation impacts adjacent sequences, then the most likely explanation for the defective phenotype of the complemented mutant strain is an unlinked and unrecognized mutation elsewhere in the genome. In this case, complementation has failed because the phenotype of the mutant strain does not derive from the introduced mutation, thus illustrating the essential role of complementation in genetic studies.

Use of published 5′ RNA-seq data to predict endogenous promoters

When designing a complementation construct it is important to incorporate sufficient sequence upstream of the complementing gene to ensure its appropriate regulation. Similarly, the genomic and transcriptional context of the gene should be taken into account, including whether or not the gene is transcribed alone or with other genes as a part of an operon. The *B. burgdorferi* 5′ end transcriptome has provided significant information for refined annotation of the *B. burgdorferi* genome, including a genome-wide map of transcription start sites and a comprehensive analysis of 5′ UTR length (Adams et al., 2017b). These data provide a valuable resource (https://lymedisease.med.ucf.edu/) for predicting endogenous promoters and putative regulatory sequence, critical for the design of effective mutagenesis and complementation approaches.

Surrogate genetics

Limitations on the genetic manipulation of *B. burgdorferi* have stimulated researchers to perform surrogate genetics in *E. coli* because of the well-developed genetic systems of the latter organism and the availability of extensively characterized mutants (Beckwith, 1991). Numerous *B. burgdorferi* genes have been expressed in *E. coli* from either their endogenous promoters or from cloning vector promoters, primarily as a source of purified recombinant protein. A number of *B. burgdorferi* genes have been used to complement *E. coli* or *Salmonella* mutants deficient in the homologous genes, which has permitted partial functional characterization of these borrelial genes (Tilly et al., 1993; Margolis et al., 1994; Knight and Samuels, 1999; Lin et al., 2001; Purser et al., 2003; Eggers et al., 2004; Liveris et al., 2004; Putteet-Driver et al., 2004; Bugrysheva et al., 2005; Jewett et al., 2011). Similarly, cloning of *B. burgdorferi* genes encoding phosphatidylcholine and phosphatidylglycerolphosphate synthase in *E. coli* permitted the characterization of these enzymatic activities (Wang et al., 2004). Expression in *E. coli* of a phage display library led to the identification of a putative integrin-binding adhesin of *B. burgdorferi* (Coburn et al., 1999; Coburn and Cugini, 2003). Surrogate genetics in *E. coli* also permitted the characterization of *B. burgdorferi* promoter elements involved in transcriptional regulation (Alverson et al., 2003; Boylan et al., 2003; Caimano et al., 2004; Eggers et al., 2004; Burtnick et al., 2007; Medrano et al., 2010). However, differences in metal acquisition and homeostasis between *B. burgdorferi* and *E. coli* have been cited as a reason why *E. coli* may not be an appropriate surrogate system for studying *B. burgdorferi* gene expression and regulation (Ouyang et al., 2010).The advances in genetic manipulation of *B. burgdorferi* described in this review suggest that the need to perform surrogate genetic experiments in other bacterial hosts may decrease in the future. However, given its complex culture medium

and slow growth rate, it is unlikely that *B. burgdorferi* will ever be as easy to manipulate as *E. coli*.

Engineered gene expression

Endogenous promoter fusions

The first tools employed to control gene expression in *B. burgdorferi* were its endogenous *flaB* and *flgB* promoters (Sohaskey et al., 1997; Bono et al., 2000; Sartakova et al., 2000). These two promoters have been used extensively to constitutively express endogenous and foreign genes in *B. burgdorferi*, and to develop new genetic markers and genetic tools, as described throughout this review. Enzootic stage-specific expression has been achieved using the *ospA* and *ospC* promoters, for tick stage and mammalian stage expression, respectively (Tilly et al., 2016; Adams et al., 2017b). Constitutive expression mediated by *flaBp* has also been utilized to study the role of *ospC*-repression in the ability of *B. burgdorferi* to evade mammalian host defenses (Xu et al., 2006). Similarly, *B. burgdorferi* containing *flaBp-ospAB* gene fusions were used to demonstrate that constitutive expression of these proteins led to rapid clearance of *Borrelia* in mice by the immune system (Strother et al., 2007). For many applications, strong gene expression driven by the *flaB* and *flgB* promoters is experimentally useful; however, overexpression of certain proteins can be limited by possible toxic effects, mis-localization and even altered function (Hayes et al., 2014). Recently, to address these challenges, constitutive promoters of low and intermediate strengths were identified from a global transcriptome data set that measured *B. burgdorferi* gene expression at multiple *in vitro* growth phases (Arnold et al., 2016; Takacs et al., 2018). This panel of cloned and validated promoters provides a range of levels of constitutively controlled expression of genes of interest (Takacs et al., 2018). Future analysis of the activities and utility of these promoters *in vivo* will be important.

Inducible gene expression

Inducible promoter systems that can regulate gene expression in a graded manner offer certain advantages over the total ablation of gene expression generated by null mutants, or constitutive expression generated by endogenous *B. burgdorferi* promoters. For example, information about the function of unknown but essential genes can be obtained through modulating gene expression and monitoring the phenotypic changes that occur when gene products become limiting. In *B. burgdorferi*, endogenous genetic switches have yet to be identified. However, well characterized genetic switches, such as the *E. coli* lactose operon (Jacob and Monod, 1961) and the *E. coli* tetracycline resistance gene of *Tn*10 (Gossen and Bujard, 1992; Bujard, 1999), have been adapted for use in *B. burgdorferi*.

lac system

The *lac* system has been adapted to experimentally regulate gene expression in *B. burgdorferi* (Blevins et al., 2007; Gilbert et al., 2007). Gilbert *et al.* (Gilbert et al., 2007) constructed the *flacp* inducible promoter by integrating the synthetic *lacO*id operator (Oehler et al., 1994) into the *B. burgdorferi flgB* promoter (Ge et al., 1997). This inducible promoter was fused to *ospC* and *rpoS* by directly incorporating *flacp* into the genome and replacing the native promoters of the respective genes (Gilbert et al., 2007). These investigators also

generated *B. burgdorferi* strains that produce high levels of the LacI repressor by fusing the *flgB* promoter to *lacI* and inserting the hybrid into lp25, a plasmid required for infectivity (Purser and Norris, 2000; Labandeira-Rey and Skare, 2001). The expression of *flacp*-controlled genes in this background is tightly regulated in response to the inducer isopropyl β-D-thiogalactopyranoside (IPTG); genomic *flacp-ospC* and *flacp-rpoS* fusions bypass the normal regulation by environmental factors (Gilbert et al., 2007). In addition, *flacp-rpoS* was used to demonstrate that RpoS was not sufficient to downregulate OspA *in vitro* (Caimano et al., 2007). This *lac* system was used *in vivo* (Gilbert et al., 2007) to further test the hypothesis that OspC was required at an early time point in infection (Tilly et al., 2006; Tilly et al., 2007). This experiment indicated that the inducible system functions *in vivo* and can be used to test genes required for infectivity (Gilbert et al., 2007).

An alternative system of gene regulation based on the *E. coli lac* operon was also shown to be functional in *B. burgdorferi* (Blevins et al., 2007). Gene regulation by the *lac* operator was demonstrated using a codon-optimized luciferase reporter (luc_{Bb}) and through expression of the endogenous *B. burgdorferi* protein BptA. In this system, the *B. burgdorferi lac* expression system consisted of a single plasmid based on the pKFSS1 shuttle vector with the T5/*lac* hybrid promoter *pQE30* from plasmid QE30 (QIAGEN); this promoter contains two *lac* operators (Blevins et al., 2007). On the same plasmid, a codon-optimized *lacI* gene was present under the control of the *flaB* promoter (Blevins et al., 2007). Adding IPTG to *B. burgdorferi* carrying this plasmid with either luc_{Bb} or *bptA* cloned under the control of the *pQE30* promoter resulted in increased expression of these gene products. This *lac* system worked relatively well in *B. burgdorferi*, but induction was incomplete, which may result from either insufficient IPTG or excessive LacI inside the cell, or weak activity of the *pQE30* promoter in *Borrelia*.

tet **system**

Like the *lac* system, the tetracycline-inducible system has been adapted for controlling gene expression in *B. burgdorferi* (Cabello et al., 2006; Whetstine et al., 2009). These approaches use hybrid tetracycline-responsive *B. burgdorferi* promoters and constitutively expressed *tetR*. Placement of the *tet* operator upstream of the −10 and −35 sequences of the *bmpA* promoter resulted in inducible, but significantly leaky expression of the target gene (Cabello et al., 2006). In contrast, the hybrid P_{ost} promoter includes the *ospA* promoter and 5′ UTR sequence with the first 19 nucleotides of the 5′ UTR replaced with the *tetO* sequence (Whetstine et al., 2009). The P_{ost} promoter, along with the constitutively expressed *tetR* gene, are engineered on a single kanamycin-resistant shuttle vector, pCRW53 (Whetstine et al., 2009). This system demonstrates little to no background expression in the absence of anhydrotetracycline (ATc) and titratable expression up to 2 μg/ml ATc. Yet, maximal P_{ost} promoter activity was not achieved, perhaps due to read through-transcription of *tetR*. In its current design, the pCRW53 tet-inducible system is limited to environments in which *ospA* is expressed and further adaptation is required for its application during mammalian infection (Whetstine et al., 2009).

These results demonstrate that gene expression can be artificially regulated in *B. burgdorferi* using either the *lac* system or the *tet* system. Modifications yielding stronger and tighter

induction of genes under the control of these inducible promoters should be possible in the future. The availability of inducible expression systems for *B. burgdorferi* has allowed isolation of conditional lethal mutants through regulated repression of a number of essential genes. (Lenhart and Akins, 2010; Groshong et al., 2012; Bandy et al., 2014; Ye et al., 2014; Chu et al., 2016; Groshong et al., 2017; Drecktrah et al., 2020). Conversely, increased expression of *rpoS* from an inducible *lac* promoter resulted in cell death, consistent with a failure to engineer over-expression of *rpoS* from the strong *flaB* promoter (Chen et al., 2013). Further optimization of these genetic switches, as well as development of endogenous genetic switches, will improve manipulation of *B. burgdorferi* gene expression *in vivo* and open a new avenue for studies of pathogenesis in Lyme borreliosis.

Random tagged mutagenesis

The use of transposon mutagenesis as a tool for bacterial genetics represents an important advance that has generally superseded chemical and radiation mutagenesis (Hayes, 2003; Bosse et al., 2006; Salama and Manoil, 2006; Burrack and Higgins, 2007). Mutagenized bacteria are easily isolated on the basis of antibiotic resistance or other selectable markers carried on the transposable element, and the mutated gene can be readily identified by routine molecular procedures (Judson and Mekalanos, 2000; Hayes, 2003; Bosse et al., 2006; Salama and Manoil, 2006; Burrack and Higgins, 2007).

Genome-wide transposon mutagenesis of *B. burgdorferi* was first demonstrated with a suicide plasmid that contained a modified *Himar1* element of the *mariner* family (Stewart et al., 2004). In this system, called pMarGent, the inverted repeats of the transposable element flank a ColE1 origin of replication and a gentamicin-resistance cassette that functions in both *E. coli* and *B. burgdorferi*, while the *Himar1* transposase is carried on a portion of the suicide plasmid that is not mobilized, and hence only transiently present in *B. burgdorferi* (Stewart et al., 2004; Stewart and Rosa, 2008). This approach yielded a saturated mutant library in a non-infectious *B. burgdorferi* clone and permitted the isolation and characterization of a large number of mutants, with random transposon insertions in the linear chromosome and all linear and circular plasmids (Stewart et al., 2004). These experiments demonstrated the strength and feasibility of transposon mutagenesis in *B. burgdorferi*.

The utility of transposon mutagenesis as a tool to study *B. burgdorferi* virulence was illustrated by using the pMarGent system to generate a limited number of transposon mutants in an infectious *B. burgdorferi* clone and subsequently assessing their ability to infect mice (Botkin et al., 2006a; Botkin et al., 2006b). However, this approach was limited due to the instability of the pMarGent plasmid in *E. coli* and the low transformation frequency of infectious *B. burgdorferi*. To reduce the possibility of recovering inactive plasmid from *E. coli* due to transposition of the gentamicin marker, a kanamycin resistance cassette was added adjacent to the transposase, creating the vector pGKT (Stewart and Rosa, 2008). However, this modification does not fully stabilize pGKT because both markers can be retained in the same cell at different genomic locations following transposition. The limitation on transposon mutagenesis imposed by *B. burgdorferi*'s low transformation frequency has been partially addressed, as in other genetic approaches, with *B. burgdorferi*

clones lacking lp56 and gene *bbe02* on lp25, thereby avoiding the R/M systems they encode (Kawabata et al., 2004; Rego et al., 2011; Lin et al., 2012; Lin et al., 2014; Lin and Gao, 2018). Lin et al. further adapted the pGKT vector for signature tagged mutagenesis (STM) by inserting eleven distinct 7-bp tags in the region between the ColE1 and inverted terminal repeat 2 (Lin et al., 2012). This molecular genetic advance allowed for generation of a genome-scale STM library of 4,479 sequence-defined mutants, which can be interrogated in groups of clones to identify genes important for *in vivo* and *in vitro* phenotypes (Lin et al., 2012).

Isolation of a random, saturated mutant library requires an insertion every 200 base pairs on average, thus a completely saturated transposon library of *B. burgdorferi* would contain at least 7,500 independent mutants, since the *B. burgdorferi* genome is approximately 1,500,00 bp (Fraser et al., 1997; Casjens et al., 2000; Salama and Manoil, 2006). Generation of a genome-wide STM library necessitated development of a multiplex, high-throughput method for plasmid content analysis of the individual clones. The Luminex plasmid analysis assay employs a combination of three multiplex PCRs using 24 primer sets specific for each of the 21 *B. burgdorferi* B31 plasmids, and Luminex xMAP technology for microfluidic bead-based detection of the *B. burgdorferi* replicons (Norris et al., 2011). This approach allows simultaneous analysis of 30 *B. burgdorferi* clones in a 96-well plate format and has been used to assess the plasmid content of 44 B31 clones and 4,464 transposon mutants in an STM library (Norris et al., 2011). To date, 434 STM mutants from the library of 4,479 unique mutants have been screened for infectivity in mice (Lin et al., 2012), resulting in the identification of infection-relevant candidate genes (reviewed in Radolf and Samuels, 2021). Initially, comparison of the input and output STM mutant pools to identify those mutants that were attenuated for infection involved a semi-quantitative Luminex-based multiplex PCR procedure (Lin et al., 2012). More recently, Tn-seq has been used to identify genes contributing to a phenotype. Using this approach, the fitness of STM mutants is determined by sequencing the genomic DNA flanking the transposon insertion sites in the entire library, before and after undergoing selection in the environment of interest (Troy et al., 2016). Tn-seq has been applied in *B. burgdorferi* to identify gene candidates important for mouse infection, survival in ticks, resistance to reactive oxygen and nitrogen species and carbohydrate utilization (Troy et al., 2016; Ramsey et al., 2017; Phelan et al., 2019), (see Radolf and Samuels, 2021), as well as to identify when and where *B. burgdorferi* encounters barriers to mouse infection (Troy et al., 2016).

STM library screens are powerful molecular genetic tools to identify candidate genes of interest. It is important to note, however, that genetic screens of this nature do not assess the fitness of a single mutant alone in the challenge environment, rather the fitness of all mutants in the input pool relative to each other. For this reason, the phenotype of a single mutant alone may differ from that in the context of a pool of other clones. Further studies, including targeted inactivation and complementation, are required to validate the roles of the implicated genes in the phenotype of interest. Despite technical advancements in the transformation frequency of *B. burgdorferi*, the challenge of achieving a saturated transposon mutant library remains daunting. The increased technical feasibility and reduced cost of whole genome re-sequencing raises the possibility for the resurgence of chemical and radiation mutagenesis approaches as alternatives for genetic screens in *B. burgdorferi*.

Reporter genes

Reporter genes encode proteins whose function, such as enzymatic activity, fluorescence or selectable phenotype, can be readily assayed or detected; they have been used extensively to monitor gene expression, to track cells in complex environments, and to determine the subcellular locations of proteins. Different types of reporters are typically better suited for distinct applications. The reporter genes currently available for *Borrelia* and their pertinent features are summarized in Table 2.

In vitro gene expression

A number of reporter genes have been used successfully as transcriptional fusions with *B. burgdorferi* promoters to study gene expression, including *cat* (chloramphenicol acetyl transferase), *luc* (luciferase), *lacZ* (β-galactosidase), and *gfp* (green fluorescent protein) (Sohaskey et al., 1997; Sartakova et al., 2000; Carroll et al., 2003; Eggers et al., 2004; Blevins et al., 2007; Hayes et al., 2010). The initial studies in *B. burgdorferi* used promoter:*cat* fusions to assay gene expression (Sohaskey et al., 1997; Sohaskey et al., 1999), but *cat* has largely been replaced by other reporter genes that are easier to use or have broader utility. β-galactosidase, encoded by the *lacZ* gene, is a widely used reporter whose activity can be easily monitored in bacteria grown in liquid and solid media without specialized equipment or radioactivity (Sambrook et al., 1989). A codon-optimized *lacZ* reporter was developed for *B. burgdorferi*, and β-galactosidase activity measured in lysates of cultured spirochetes carrying *lacZ* transcriptional fusions with a standard Miller assay (Hayes et al., 2010). The omission of phenol red from BSK medium facilitates detection of *lacZ*-expressing (blue) *B. burgdorferi* colonies in plates containing X-Gal (Hayes et al., 2010). Use of a *lacZ* reporter in a blue-white colony screen confirmed the identity of a negative regulator of *ospC* encoded on linear plasmid lp17 (Sadziene et al., 1993; Sarkar et al., 2011; Hayes et al., 2014). The availability of *lacZ* further expands the repertoire of tools for investigating mechanisms of gene regulation in *B. burgdorferi*.

Whereas cat and *lacZ* reporters are routinely used to quickly measure gene expression in a population of cells, fluorescent protein reporters offer other advantages, such as directly monitoring gene expression at the individual cell level by microscopy or flow cytometry. Green fluorescent protein (GFP) was the first fluorescent protein reporter used to monitor gene expression and identify promoter elements in *B. burgdorferi* (Carroll et al., 2003; Eggers et al., 2004; Falkow, 2004; Valdivia et al., 2006; Srivastava and de Silva, 2008). GFP signal can be influenced by temperature, pH and oxygen concentration, but is relatively stable, which limits its utility for detecting rapid changes in gene expression (Valdivia et al., 2006). An expanded range of alternative fluorescent reporter genes that have been expressed in *B. burgdorferi* include yellow fluorescent protein, *yfp*; cyan fluorescent protein, *cfp* (Eggers et al., 2002); monomeric red fluorescent protein, *mRFP* (Schulze and Zuckert, 2006) and dTomato (Lee et al., 2010). Recently, a number of *B. burgdorferi* codon-optimized reporters have been developed, including CFP variants, mCerulean and msfCFP; GFP variants, mEGFP and msfGFP; YFP variants, mCitrine and msfYFP and a monomeric improved variant of mRFP1, mCherry (Takacs et al., 2018). In addition, an infrared fluorescent protein (iRFP) reporter was developed. iRFP is an extrinsically fluorescent

protein that covalently binds biliverdin as the fluorophore (Filonov et al., 2011). Growth of *B. burgdorferi* expressing iRFP in medium supplemented with the biliverdin cofactor results in near-infrared fluorescence of the cells (Takacs et al., 2018). In addition to *in vitro* studies, iRFP may be useful for *in vivo* live imaging of infected mice and/or *ex vivo* imaging of infected tissue explants, as described elsewhere in this review, due to the high excitation light penetrance and low tissue auto-fluorescence in the infrared region of the spectrum. Further, the endogenous levels of biliverdin in animal tissues are predicted to be sufficient for maximal fluorescence of iRFP-expressing *B. burgdorferi* (Takacs et al., 2018), although this has yet to be tested experimentally. Combinatorial imaging studies demonstrated that CFP variants can be co-imaged with mCitrine, mCherry and iRFP, and GFP variants can be co-imaged with mCherry and iRFP, suggesting the possibility for simultaneous imaging of up to four proteins in the same spirochete (Takacs et al., 2018).

Subcellular protein localization

In addition to their utility as transcriptional fusions for gene expression studies, translational fusions of fluorescent proteins represent important tools for cell biology, as described in greater detail in Radolf and Samuels (2021). Monomeric red fluorescent protein (mRFP) has been used in this capacity to investigate the lipoprotein export pathway of *Borrelia* (Schulze and Zuckert, 2006) and to develop a FACS-based lipoprotein localization screen (Kumru et al., 2010). More recently, an mCherry fusion protein was employed in a microscopy-based approach, coupled with quantitative image analysis, to investigate the dynamic cellular localization of an envelope protein of *Borrelia* (Takacs et al., 2018). A key feature of mRFP and mCherry as fusion partners for protein localization studies is that they continue to fluoresce outside of the cytoplasm, which earlier variants of GFP did not allow (Schulze and Zuckert, 2006). However, this previous limitation has been overcome with super-folding variants of GFP (sfGFP) that maintain fluorescence in periplasmic and extracellular locations (Pedelacq et al., 2006; Dinh and Bernhardt, 2011). A shortcoming of fluorescent proteins as translational fusions is their relatively large physical size, which can hinder the function, folding or localization of the protein to which they are fused. This potential drawback can be avoided with an alternative fluorescent labeling approach that entails tagging a cellular protein with a small tetra-cysteine motif that can bind fluorescent biarsenical dyes (Griffin et al., 1998). This approach has been used to label membrane proteins in *B. burgdorferi* and while avoiding the size constraints of fluorescent proteins, it is considerably less sensitive, requires a labeling step and can be limited by non-specific binding of the fluorescent dye (Hillman et al., 2019).

In vivo tracking and imaging

Beyond applications with spirochetes grown *in vitro*, reporter genes are important tools for detecting *B. burgdorferi* in ticks and mice (see Radolf and Samuels, 2021, for an in-depth review). GFP has permitted study of gene expression of individual spirochetes in ticks (Bykowski et al., 2006; Miller et al., 2006), and this reporter was used to visualize the interaction of the spirochete with the mouse vasculature in real time with high resolution imaging (Moriarty et al., 2008). Furthermore, GFP-expressing *B. burgdorferi* have been visualized in the intact skin of living mice and time-lapse images taken early after infection have allowed analysis of spirochete motility *in vivo* (Sultan et al., 2015; Novak et al., 2016).

GFP-expressing *B. burgdorferi* were used to track spirochete migration from an infected mouse to a feeding tick (Bockenstedt et al., 2014) and spirochete dissemination within ticks (Dunham-Ems et al., 2009).

Further expansion of *in vivo* live imaging of *B. burgdorferi* during infection has come with adaptation of a codon-optimized luciferase gene (luc_{Bb}) from the firefly *Photinus pyralis* for use in *B. burgdorferi* (Blevins et al., 2007). Luciferase activity of the firefly luc_{Bb} reporter, which requires an exogenous substrate and is measured with a luminometer, was first used to monitor the activity of endogenous *Borrelia* promoters and an ITPG-induced *lac* expression system (Blevins et al., 2007), as described above. Subsequently, *in vivo* bioluminescent imaging of mice infected with *B. burgdorferi* constitutively synthesizing luciferase has been used for temporal and spatial tracking of spirochete infection at the population level (Hyde et al., 2011a; Chan et al., 2015; Wager et al., 2015; Skare et al., 2016; Adams et al., 2017b; Hyde and Skare, 2018). The luc_{Bb} gene, driven by the strong constitutive *flaB* promoter and expressed on a multi-copy shuttle vector, allows detection of bioluminescent *B. burgdorferi* in live mice as early as three days post-inoculation (Adams et al., 2017b). This technique works well using both BALB/c and C3H/HeN mice (Hyde et al., 2011a; Adams et al., 2017b). The relative luminescence units (RLUs) detected by imaging mice infected with bioluminescent *B. burgdorferi* correlates with spirochete load in tissues of the same mice, as measured by quantitative PCR, allowing assessment of the ability of *B. burgdorferi* mutant clones to survive and disseminate in a live animal over time (Hyde et al., 2011a; Wager et al., 2015).

Quantifying *in vivo* gene expression

Application of *in vivo* bioluminescent imaging for quantitation of *B. burgdorferi* promoter activity as a means to measure expression of target genes during infection is less well developed. Currently this approach involves normalizing the luminescence signals of mice infected with *B. burgdorferi* carrying luc_{Bb} fused to the target gene promoter, with those of other mice infected with *B. burgdorferi* constitutively expressing luc_{Bb}, and subsequent qPCR analysis to demonstrate that all mice carry equivalent bacterial loads at the endpoint of the study (Skare et al., 2016). A dual reporter system that allows simultaneous measurement of the luminescence signal of the target promoter and a control constitutive promoter from the same population of spirochetes would provide a more straightforward and robust method to quantitate gene expression during infection. The first dual luciferase reporter system of *B. burgdorferi* was recently developed (Adams et al., 2017a). For this reporter system, constitutive expression of the *B. burgdorferi* codon-optimized *Renilla reniformis* (sea pansy) luciferase gene (P_{flaB}-$rluc_{Bb}$) allows normalization of the activity of a second promoter fused to the *B. burgdorferi* codon-optimized firefly luciferase ($fluc_{Bb}$) gene on the same shuttle vector plasmid. The bioluminescence signals of the two reporters are distinguishable due to their respective specificities for different substrates, coelenterazine and luciferin. This dual luciferase system is a simple and robust approach for quantitative analysis of *B. burgdorferi* promoter activity under *in vivo* growth conditions and in infected ticks (Adams et al., 2017a). Application of this dual reporter system to *B. burgdorferi* promoter activity during mammalian infection remains a challenge, however, because coelenterazine demonstrates high background fluorescence in the mouse that was not

circumvented through extensive modification of experimental parameters (Adams et al., 2017a). Future development of a dual reporter system for *in vivo* live imaging of *B. burgdorferi* infection may include a combination of green- and red-shifted luciferase enzymes (Branchini et al., 2005) or application of iRFP (Takacs et al., 2018), as discussed above.

Identifying *in vivo* promoters

Reporter genes are not limited to those whose expression can be measured as an enzymatic activity, fluorescence, luminosity or antibiotic resistance. A gene that is required for survival in a specific environment can also be used as a reporter of promoter activity. This concept is the basis of *in vivo* expression technology (IVET), a powerful and versatile method for genome-wide identification of promoters that are active in a host environment, by complementation of an auxotrophic mutation through gene fusion (Mahan et al., 1993). IVET has been adapted for *B. burgdorferi* using the *pncA* gene (Ellis et al., 2013; Casselli and Bankhead, 2015), which encodes a nicotinamidase activity that is required during infection of a mammalian host (Purser et al., 2003). The *pncA* promoter trap provides strong selection for *in vivo*-active promoters, as only those clones from the IVET library that express *pncA* during infection will survive in the mouse. Moreover, when expressed, *pncA* partially complements the phenotype of *B. burgdorferi* lacking virulence plasmid lp25 (on which *pncA* is encoded), allowing the IVET library to be generated in a more readily transformable genetic background, as described above (Lawrenz et al., 2002; Purser et al., 2003; Ellis et al., 2013). The initial IVET method in *B. burgdorferi* used the *pncA* reporter alone and identified promoters that were active both *in vitro* and during infection, as well as those that were specifically active during infection (Ellis et al., 2013; Adams et al., 2017b). This approach identified 233 unique infection-active promoters across a majority of the *B. burgdorferi* replicons (Ellis et al., 2013; Adams et al., 2017b). These sequences have led to the identification of novel genes critical for *B. burgdorferi* infectivity (Jain et al., 2015; Showman et al., 2016), as well as the discovery of previously unannotated intergenic, intragenic and antisense transcripts throughout the *B. burgdorferi* genome (Adams et al., 2017b).

In vivo phenotype

An aspect of genetic studies with *B. burgdorferi* that requires recognition (but is not unique to this bacterial pathogen) is the phenotype of the organism and the context in which gene function is addressed. Cultured spirochetes look and respond quite differently from genetically identical organisms present in infected ticks or mice (Iyer et al., 2015). Thus *in vitro* assays may not accurately reflect or predict the *in vivo* function of a particular *B. burgdorferi* gene product because other differentially synthesized bacterial proteins could mask or expose its function. There is no ready solution to this potential problem, but interpretation of results obtained from *in vitro* assays should include this caveat. Propagation of spirochetes in implanted chambers in the peritoneal cavity of rats results in bacteria exhibiting a partially host-adapted state, as assessed by altered patterns of protein synthesis and gene expression (Akins et al., 1998; Revel et al., 2002; Brooks et al., 2003; Caimano,

2005) and presents a somewhat more relevant source of bacteria for testing the *in vitro* properties of mutants.

Offsetting the inefficiencies and challenges of genetic studies in *B. burgdorferi* is the availability of relevant animal models in which to analyze mutants. Rodents are natural reservoir hosts, and both ticks and mice can be experimentally infected, making the entire infectious cycle amenable to investigation. Another publication (Radolf and Samuels, 2021) describes the animal models of Lyme disease in greater detail. Several parameters of the in *vivo* model that merit attention with respect to mutant characterization are infectious dose, mode and route of challenge, mouse strain, number of animals and tissues analyzed, quantitation of tissue burden and gene expression, and the statistics used to evaluate the data. Competitive index may be a useful parameter for discovery and quantitation of more subtle phenotypes (Bestor et al., 2012; Dulebohn et al., 2013; Arnold et al., 2015). Experimental design typically is influenced by the mutant phenotype and the focus of the investigation.

When designing, executing and interpreting in *vivo* genetic screens that use a highly complex inoculum (such as a saturated library of mutants) in which the ability to be recovered (or not) is a defining trait (such as IVET or Tn-seq), it is important to keep in mind the population dynamics of *B. burgdorferi* infection. Population bottlenecks during infection have been experimentally demonstrated using both Tn-Seq and genetic bar-coding approaches (Troy et al., 2013; Rego et al., 2014). A significant population bottleneck was found to occur at the site of infection, due in part to early innate immune responses (Troy et al., 2013; Casselli and Bankhead, 2015). Population bottlenecks were also detected during persistent infection of mice, as well as during spirochete acquisition and transmission by ticks (Rego et al., 2014; Phelan et al., 2019). These data highlight the potential misinterpretation of a mutant phenotype in the context of a mixed infection (*in vivo* genetic screen or competition assay) because clones can undergo stochastic elimination at a bottleneck independently of their genotype.

Genetic manipulation of relapsing fever spirochetes

A significant advance in the *Borrelia* field has been the development of a genetic system for the relapsing fever (RF) spirochetes (Battisti et al., 2008; Fine et al., 2011; Guyard et al., 2013; Lopez et al., 2013; Fine et al., 2014; Raffel et al., 2014; James et al., 2016; Krishnavajhala et al., 2017; James et al., 2018; Jackson-Litteken et al., 2019). The availability of genetic tools and complete genomic sequences for multiple RF species (Lescot et al., 2008; Elbir et al., 2012; Miller et al., 2013; Barbour and Campeau Miller, 2014; Barbour, 2016; Wilder et al., 2016; Elbir et al., 2017; Kuleshov et al., 2020) has permitted investigation of genetically modified RF spirochetes in their respective tick vectors or rodent hosts, and informative comparisons with other *Borrelia*, as detailed in Radolf and Samuels (2021). As with LD spirochetes, there is considerable variation among RF strains in the efficiency with which they can be grown, transformed or recovered as colonies in solid medium (Raffel et al., 2018). A noteworthy feature of genetic studies with RF spirochetes is retention of infectivity following prolonged cultivation (Lopez et al., 2008), suggesting a more stable segmented genome or a stronger requirement for plasmid-encoded functions during *in vitro* growth, relative to the LD *Borrelia*. Additional research is

needed to determine if this attribute is true for all RF strains and clones, and how it is influenced by media and culture conditions (Raffel et al., 2018). Another advantage of RF *Borrelia* for genetic studies is the relatively high density they reach in the blood of an infected mouse during a relapse, permitting direct visualization and quantitation of *in vivo* organisms, and sufficient material for downstream analyses.

The genetic approaches and tools developed for the LD spirochetes provided a strong foundation for molecular genetic manipulation of the closely related RF spirochetes. Generation of the first site-directed allelic-exchange deletion mutant in *Borrelia hermsii* was reported in 2008 (Battisti et al., 2008). Furthermore, this work included a method for genetic transformation by electroporation and isolation of individual *B. hermsii* clones in liquid medium by limiting dilution in 96-well plates. In addition, antibiotic resistance cassettes and a *B. hermsii* shuttle vector for *trans*-complementation and expression studies were described (Battisti et al., 2008). Further expansion of the genetic tools for RF spirochetes came with the generation of an infectious *B. hermsii* clone that constitutively expresses GFP, allowing visualization of fluorescent spirochetes both *in vitro* and *in vivo* (Fine et al., 2011). The methods for genetic manipulation of *B. hersmii* have been applied successfully to another RF spirochete, *B. turicatae* (Lopez et al., 2013; Krishnavajhala et al., 2017). Most recently, a method for reliable growth of *B. hermsii* and *B. turicatae* in solid medium has been developed, allowing isolation of single colonies following genetic manipulation, as well as enumeration of colony forming units from both *in vitro* and *in vivo* samples (Raffel et al., 2018). The application of reverse genetics to the RF spirochetes has opened the door to broad investigation of these tick-borne pathogens (Raffel et al., 2014; James et al., 2016, 2018; Jackson-Litteken et al., 2019; Schwan et al., 2020).

Conclusion

The genetic toolbox for *B. burgdorferi* continues to grow, expanding the spectrum of approaches available for investigating the genetic mechanisms driving the biology of this fascinating pathogen. An increased understanding of the R/M systems that limit transformation efficiency has resulted in *B. burgdorferi* clones with enhanced capacity for transformation while retaining infectivity. These *B. burgdorferi* clones have been critical for improved recovery of targeted mutant and complemented clones, as well as the generation of genome-wide mutant and promoter-trap libraries. The development of a wide array of new reporter genes have opened the door for novel microscopy, flow-cytometry, genetic screening, *in vivo* bioluminescence, and *in vivo* selection assays. Moreover, new constitutive and inducible promoters allow for various levels of engineered expression of genes of interest. Molecular genetic approaches pioneered in *B. burgdorferi* have facilitated the development of tools for genetic manipulation and investigation of the closely related RF spirochetes. Transformation, plating in solid medium, reverse genetics, and florescence-tagging of *B. hermsii* and *B. turicatae* are critical advances for understanding these medically important spirochetes. In sum, although there are still challenges and obstacles to overcome, significant strides have been made in *Borrelia* genetics, and persistent innovation and creativity will continue to increase and enhance the genetic tools available for the study of LD and RF spirochetes.

Acknowledgements

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Table 1.

selectable markers for *Borrelia*

ANTIBIOTIC RESISTANCE CASSETTES

gene	promoter	origin	resistance	selection (µg ml⁻¹)	comments	reference
gyrB301	Native (*gyrB*)	*B. burgdorferi* chromosome	coumermycinA₁	0.2–5	high rate of recombination at endogenous *gyrB* locus; pleiotropic effects	(Samuels et al., 1994)
aphI	*flgB, flaB*	*E. coli* Tn*903*	kanamycin	200–400	first widely used selectable marker in *Borrelia*	(Bono et al., 2000; Battisti et al., 2006)
ermC	native	*Staphylococcus aureus*	erythromycin	0.03–0.06	some *Borrelia* strains are resistant to erythromycin	(Sartakova et al., 2000)
aacC1	*flgB, flaB*	*Pseudomonas aeruginosa* Tn*1696*	gentamicin	40	selection with this marker can be variable in E *coli*	(Elias et al., 2003)
aadA	*flgB, flaB*	*Shigella flexneri* plasmid R100	streptomycin*a*	50	*a*confers resistance to both streptomycin and spectinomycin	(Frank et al., 2003; Jewett et al., 2007a)
bsd_Bb	*flgB*	*Aspergillus terreus*	blasticidin	10–160	codon-optimized for *Borrelia*	(Takacs et al., 2018)
hph_Bb	*flgB*	*E. coli*	hygromycin	0.2–0.3	codon-optimized for *Borrelia*	(Takacs et al., 2018)

[a]Streptomycin is used for selection in *B. burgdorferi* because spontaneous resistance to spectinomycin is high, whereas spectinomycin is used *in* E *coli*, because many strains are resistant to streptomycin.

Table 2.

reporter genes for *Borrelia*

REPORTER	APPLICATION(S)	LIMITATION(S)	REFERENCE(S)
Borrelia burgdorferi			
cat (chloramphenicol acetyl transferase)	*in vitro* gene expression	requires radioactivity or ELISA	(Sohaskey et al., 1997; Alverson et al., 2003)
lacZ (β-galactosidase)	*in vitro* gene expression; colorimetric detection in liquid and solid medium; easy blue/white screening	requires X-gal substrate	(Hayes et al., 2010)
FLUORESCENT REPORTERS (listed below)	*in vitro* and *in vivo* gene expression; cellular localization of fusion proteins; *in vivo* imaging of individual spirochetes in ticks and mice	fluorescence parameters vary among reporters and alleles; not all reporters tested for *in vivo* applications	
GFP, mEGFP, msfGFP (Green Fluorescent Protein)			(Carroll et al., 2003; Eggers et al., 2004; Moriarty et al., 2008; Dunham-Ems et al., 2009; Takacs et al., 2018)
YFP, mCitrine, msfYFP (Yellow Fluorescent Protein)			(Eggers et al., 2002; Takacs et al., 2018)
CFP, mCerulean, msfCFP (Cyan Fluorescent Protein)			(Eggers et al., 2002; Takacs et al., 2018)
mRFP, mCherry (Red Fluorescent Protein)			(Schulze and Zuckert, 2006; Takacs et al., 2018)
dTomato (Orange Fluorescent Protein)			(Lee et al., 2010)